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DYNAMICS AND MECHANISMS OF DNA METHYLATION REPROGRAMMING

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ABSTRACT

The molecular profiling of DNA and histone modifications in multiple in vitro and in vivo systems has enabled unique explorations of potential epigenetic regulatory pathways. An important question is whether these epigenetic pathways drive development or are subservient to genetically encoded signaling pathways? Broad changes in epigenomic profiles are a crucial observation in many mammalian developmental stages and are thought to result from the action of key signaling/gene regulatory networks. In this review, we will introduce the epigenetic basic principles and focus on how the study of DNA modification dynamics has contributed to our understanding of early development and the hypothesis that epigenome dependency is exquisitely linked to cellular states.

Keywords: DNA methylation reprogramming, embryogenesis, stem cells, gene regulatory networks

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2.1 INTRODUCTION

The potential relevance of epigenetics is now well appreciated in multiple biological contexts; embryo and germline development, tissue-specificity and cellular reprogramming, carcinogenesis and cancer genetics, neuronal and behavioral studies, nutrition and metabolism, disease studies and longevity, population and livestock studies and finally, plant development¹. This is a late twentieth and early twenty-first century highpoint for a concept that was promulgated by Conrad Waddington to resolve the paradox of cellular differentiation in organisms: how can embryological cells with similar genetic material differentiate into multiple and distinct cell types^{2, 3}? The fields of developmental and stem cell biology in plant and animal studies have led the way in demonstrating that signaling pathways, homeostasis mechanisms and gene regulatory networks organize the progression from a fertilized egg through embryogenesis and organogenesis, into a fertile adult.⁴⁻⁸ On this fundamentally genetic basis of development and disease, a differential contribution by parental genomes in mammals ensures successful developmental outcomes. This was identified as being due to parentally imprinted loci by a combination of classical and molecular genetic methodologies and represents one of the early examples of epigenetic regulation in mammals.⁹⁻¹²

Conrad Waddington introduced the term “epigenetics” in 1942, combining the concepts of “epigenesis” (the development of an organism) and “genetics” (the study of heredity of trait variation). Defining epigenetics as “the whole complex of developmental processes” connecting genotype to phenotype, he further used the word “epigenotype” to describe the biological networks linking disturbances in genotype to observable abnormal phenotypes.³ As an embryologist, Waddington’s definition of epigenetics was centered around the process of development and morphogenetic heritability. The definition of epigenetics has itself matured and been extended over time to give us a generally accepted modern molecular definition of “the study of stable changes (through cell division) in gene expression that are not explained by changes in DNA sequence”.¹³ In other words, epigenetics is the scientific discipline studying how, in the absence of genetic changes, phenotypic traits in cell types and organisms can be set up and maintained. An impressive example of epigenetics in action can be found during mouse embryogenesis. Epigenetic modifications are dramatically remodelled to help orchestrate the eventual differentiation of a fertilized zygote into an organization of highly specialized cells that will eventually constitute the whole adult organism.^{14, 15} This concept is illustrated in Waddington’s often-cited model of the “epigenetic landscape”, which depicts the apparently irreversible fate decisions that a cell is required to make during development. These decisions are underpinned by the complex networks of gene expression defining the pathways that the cell will utilize during its self-regulating progression through development.^{16, 17} Of course we now know that these differentiated states can be reprogrammed to earlier embryonic identities via other trajectories that are mediated either by nuclear transfer of somatic cells into enucleated oocytes, or the forced expression of pluripotency factors in a differentiated cell.^{18, 19} This reversal is associated with characteristic changes in epigenetic states that imitate embryonic profiles.^{20, 21} Epigenetic processes are utilized in multiple organisms including plants, bacteria, insects and animals.^{7, 22, 23} Our focus here will be mainly on mammalian studies.

An array of molecular mechanisms are known to be linked to epigenetic regulation of the genome; impacting on gene expression and organization of higher order chromatin structures, maintenance of cellular identity, preservation of

genome integrity, metabolism, and response to environmental damage.²⁴ The best studied of these involve the direct covalent modification of DNA and of histones, the proteins around which DNA is wrapped, in the various levels of chromatin organization that organize and package genetic information in the cell.^{25–27} Research into these two epigenetic mechanisms progressed separately throughout the 1970s and 1980s, with the term epigenetics only being adopted to describe the collective research of these chromatin modifications in the 1990s (with less stress on the inheritance of these modifications during cell division), and a rapid increase in research under this term ensuing from the year 2000.²⁸ In recent years, there has also been increasing evidence uncovering the importance of non-coding RNAs (ncRNAs) in epigenetic regulation of gene expression and maintenance of cell identity, through their interplay with chromatin.^{29, 30}

2.2 DNA METHYLATION MACHINERY

DNA methylation is an epigenetic modification involving the covalent addition of a methyl group from the methyl donor S-adenosyl methionine (SAM) to cytosine by DNA methyltransferases (DNMTs). 5-methylcytosine (5mC) is primarily found in the context of CpG dinucleotides that are symmetrically methylated on double stranded DNA in animal and plant genomes.^{25, 26} In 1975 this modification was described for the first time as an epigenetic mark in mammalian model systems, which could be established de novo, or could be stably inherited through somatic cell divisions and potentially maintain gene expression patterns following mitosis.^{31–33} The association with gene silencing made in these key papers was further established by studies showing that in vitro methylated DNA is transcriptionally inactive following transfer into *Xenopus* oocytes or mammalian cell lines, functionally linking DNA methylation to transcriptional repression.^{34, 35} This contribution to gene silencing was found to be enacted partly through the formation of transcriptionally inactive chromatin, promoting stable maintenance of gene expression signatures.³⁶ These findings uphold the view that a fundamental role of DNA methylation involves the formation of a less accessible chromatin structure in concert with the heritable maintenance of this altered state. However, the relationship between DNA methylation and gene silencing is proving to be more complex and sometimes indirect.^{37, 38} The conversion of cytosine to 5mC results in the generation of a relatively stable mark that is present at similar levels across most adult tissues. This is in contrast to 5-hydroxymethylcytosine (5hmC), a derivative of 5mC with potential roles in DNA demethylation and regulation of gene expression.³⁹ However, immunohistochemical studies with 5mC specific antibodies indicated that in early mouse development and during germline specification, DNA methylation can be dynamically programmed.^{40–42} This has been confirmed by high resolution sequencing analyses, which also found distinct DNA methylation patterns among different tissue types, between individuals, during ageing and disease states.^{6, 43–51} Differential DNA methylation at enhancer elements, with concurrent changes in histone modifications and transcription factor binding, can occur at the level of cells, tissues and individuals.^{43, 52, 53} In addition, altered methylation profiles at regulatory elements are a feature of many diverse cancer types.^{54, 55} The potential molecular mechanisms underlying this dynamic regulation have been further elucidated in recent years with the discovery of a potential demethylation pathway involving 5mC derivatives that are generated by methylcytosine dioxygenases.^{56–58} The maintenance methyltransferase DNMT1 and its cofactors (e.g., UHRF1, PCNA) have been classically considered

responsible for the perpetuation of DNA methylation during cell divisions, whereas de novo DNA methylation is initially established in mouse development by a combination of the de novo methyltransferases, DNMT3A and DNMT3B, acting in concert with their non-catalytic cofactor DNMT3L (Fig. 2.1A).^{59–62} DNMT3L itself is essential during germ cell development to ensure that endogenous retrotransposons are inactivated.⁶³ Recently a new isoform, DNMT3C, was identified in mice that is essential for retrotransposon methylation and repression in the mouse male germline.⁶⁴ Its inactivation affects mouse fertility, but it is not normally expressed in ES cells.⁶⁵ Somatic patterns of DNA methylation participate at multiple levels (locus specific, genome wide and indirectly) and in many developmental processes, including X-chromosome inactivation, genomic imprinting, retrotransposon silencing, gene repression and genome stability.^{37, 47, 66–69} Both DNA strands are symmetrically methylated at CpGs during replication, as hemi-methylated DNA is a potent substrate for the maintenance DNA methyltransferase, DNMT1, responsible for perpetuating the parental pattern.⁷⁰ Genome-wide profiling demonstrates that methylated CpG (MeCpGs) are pervasive throughout mammalian genomes, with the exception of discrete GC rich non methylated CpG “islands” (CGI). CGIs are stretches of DNA approximately 1000bp in length that have an elevated G + C content, little CpG depletion, and are normally free from DNA methylation.^{71–73} Up to 60% of human genes are associated with CGIs and predictions suggest that the human genome contains more than 25,000 CGIs with a slightly lower number (23,000) in the mouse genome. Most CGIs are associated with the 5' ends of genes, including the promoters of housekeeping genes, as well as a proportion of tissue-restricted genes and developmental regulator genes (Fig.2.2).^{72,74, 75} These regulatory landmarks are also associated with initiation sites of DNA replication.^{76, 77} The protection mechanisms that prevents de novo methylation of promoter associated CGIs maybe DNA-encoded and evolutionarily conserved. The binding of transcription factors may render CGIs resistant to the DNMTs either sterically or by promoting a chromatin environment that is refractory to DNA modification.⁷⁸ For example, unlike active X counterparts, promoter CGIs located on the inactive X chromosome in females readily acquire DNA methylation.⁷⁹ The occurrence of CGI promoter methylation can impair transcription factor binding and result in stable silencing of gene expression.⁷² Profiling experiments in mice indicate that a high proportion of autosomal methylated CGI genes correspond to genes normally expressed in early development and the germline, where they can have posttranscriptional roles in suppressing transposon activity during periods of DNA hypomethylation.^{47, 66, 67, 80} The phenomenon of aberrant promoter CGI hypermethylation is recognized as a hallmark of cancer, which in some cases, but not all, may lead to transcriptional inactivation of potential tumor suppressor genes in response to signaling cues.⁵⁴ How DNA methylation deposition at regulatory sequences interferes with transcription networks is complex but different pathways have been proposed. One pathway involves the recruitment of methyl CpG-binding proteins (MeCPs) that can target chromatin modifying activities that promote gene silencing.^{37, 81} However, inactivation of various MeCPs (including in a triple combination) has not been linked with global changes in gene expression or with regulation of well-characterized methylation-dependent genes such as *Dazl* and *Tex19.1*.^{67, 82} A more plausible model is the direct interference of transcription factor activator binding by 5mC modified bases. When tested by high-throughput screening, the binding of 1453 factors, out of approximately 1500 tested, was affected by methylated

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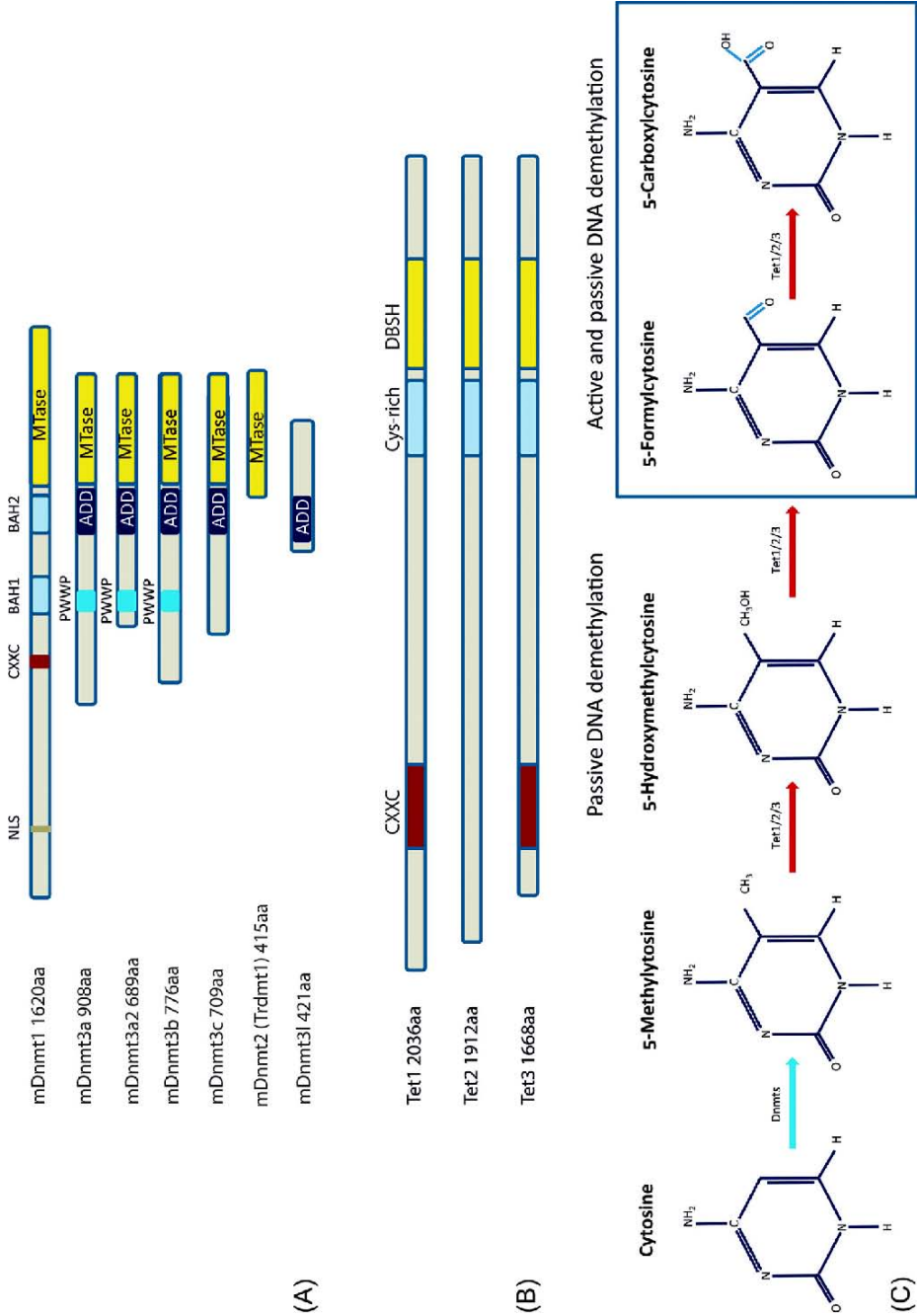


FIG. 2.1

(A) Domain structure of DNA methyltransferases. The C-terminal catalytic methyltransferase domains (MTASE) are shown (Yellow). The NLS (nuclear localization signal), the CxxC domain (which interacts with unmethylated CpGs) and two Bromo-Adjacent Homology (BAH) domains are shown for DNMT1. The DNMT3 family consists of DNMT3A, DNMT3A2, DNMT3B, DNMT3C and DNMT3L. The N-terminal regulatory domains of DNMT3A, DNMT3A2 and 3B contain the Pro-Trp-Trp-Pro (PWWP) domain important for DNA binding as well as the ADD, a PHD-like zinc finger domain important in targeting. DNMT3C is germline specific and does not contain a PWWP domain, but the catalytic methyltransferase domain is conserved. DNMT2, tRNA methyltransferase (Trdm1) lacks regulatory domains and does not significantly methylate mammalian DNA in vivo. The cofactor DNMT3L lacks the PWWP domain as well as an active catalytic domain. (B) Schematic representation of TET proteins. All TET proteins contain at the C-terminal a DSBH domain consisting of eight β -strands forming two four-stranded antiparallel β -sheets typical of the 2-oxoglutarate (2OG)-and Fe(II)-dependent oxygenase superfamily. A cysteine-rich region (Cys-rich region) is also present upstream of the DSBH domain in all TET proteins. Together, the DSBH and Cys-rich domains represent the catalytic domain of TET proteins. A CXXC zinc finger domain is present at the N-terminal of both TET1 and TET3 proteins whereas TET2 lacks this potential DNA targeting domain. Cytosine (C) is converted to 5-methylcytosine (5-mC) by DNMT proteins. 5-mC is converted to 5-hydroxymethylcytosine (5-hmC) by TET proteins, which can be relatively stable in the genome. 5hmC can be reiteratively converted to 5-formylcytosine (5-fC) and 5-carboxycytosine (5-caC) by TET proteins. 5-hmC, 5-fC and 5-caC are hypothesised to function in replication-dependent passive demethylation pathways, whereas 5fC and 5caC may participate in active demethylation pathways that incorporate DNA repair pathways to replace modified bases with cytosine (C).

CpGs in their cognate binding sites.⁸³ However, 47 factors could also bind to methylated target sequences and some TFs exhibit specific binding activity to methylated and unmethylated DNA motifs of distinct sequences. In addition, it is possible that depending on the cellular context, inhibitory effects of DNA methylation can be overridden without demethylation of target promoters suggesting that certain activating factors can overcome the inhibitory effects of DNA methylation.^{84, 85} This could shift primary control of transcriptional states towards gene-regulatory networks (GRNs), with epigenetic mechanisms acting downstream to reinforce initial GRN-mediated decisions.^{5, 86} A third possible mechanism is that DNA methylation may influence nucleosome positioning at promoter regions to affect transcription initiation.^{87, 88}

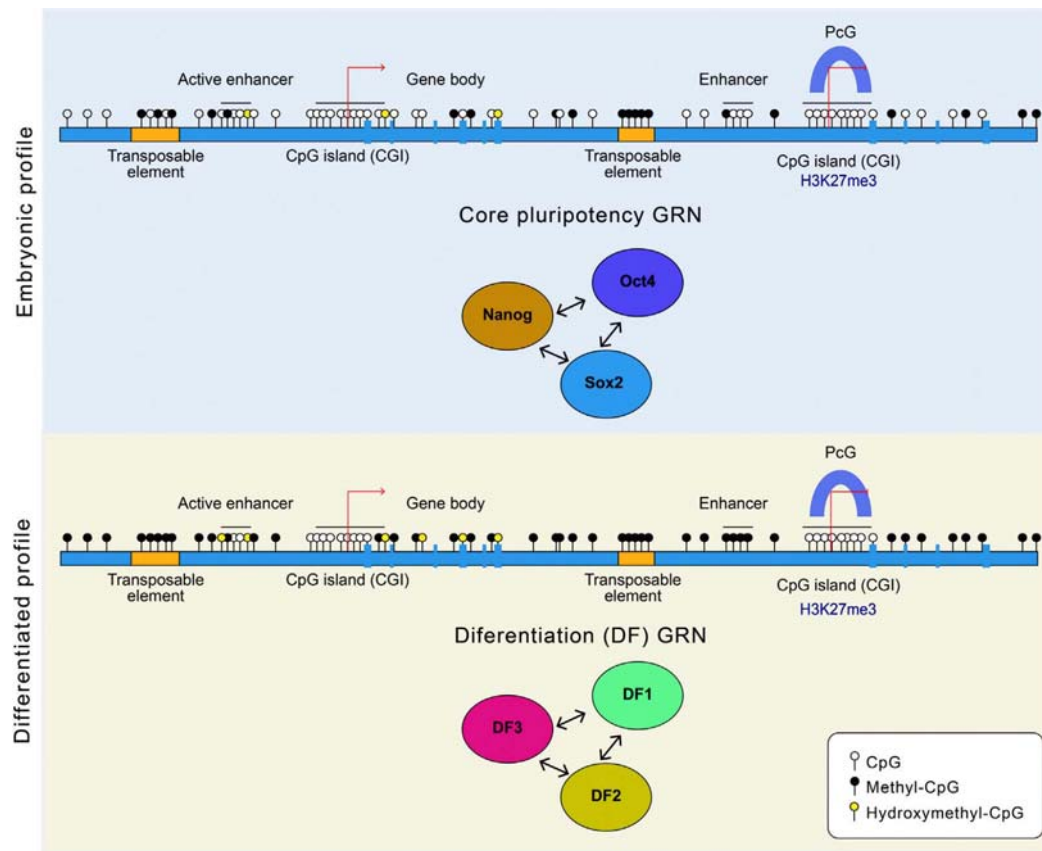


FIG. 2.2

Depiction of a generalised DNA methylation landscape in early embryonic and differentiated cells. In embryonic cells, the pluripotency gene regulation network (GRN) drives self-renewal and development with a reduced to absent dependency on DNA methylation. Active and inactive CpG island genes can be distinguished by the absence or presence of the Polycomb Regulated Complex (PRC) 2-directed trimethylation at lysine 27 on histone H3 (H3K27me3). In differentiated cells, DNA methylation is necessary to repress transposon activation, reinforce tissues states and direct differentiation factors (DFs) forming unique gene regulatory networks (GRNs). In differentiated cells, 5-hydroxymethylcytosine can mark active enhancers and is enriched at the gene bodies of expressed genes.

It should be noted that DNA methylation profiling has been used extensively in reprogramming and disease studies to chart changes in cell state, and can additionally be linked to physiological processes, such as ageing and metabolism.^{20, 44, 51, 53, 89–95}

Differentially methylated promoters associated with gene inactivation in different tissue types have been identified, but these appear to correspond to a remarkably small number of genes that are normally expressed in the germline.^{66, 67}

On the other hand, most silent nonmethylated CGI genes are associated with a histone-repressive modification profile that is dependent on Polycomb Repressive Complexes 1 and 2, which are responsible for adding a ubiquityl moiety to histone H2A at Lys119 (H2AK119ub1; PRC1) and the addition of one to three methyl groups to histone H3 at Lys27 leading to H3K27me3 (PRC2), respectively, at silenced promoters.^{71, 96}

2.3 DNA DEMETHYLATION

DNA methylation had long been considered to be a relatively stable epigenetic modification until evidence demonstrated that this modification may be more dynamic and complex than previously thought. In theory, loss of DNA methylation, or DNA demethylation can occur through passive or active processes. Two basic mechanisms leading to DNA demethylation have been proposed: (A) a passive mechanism through which remethylation of hemimethylated substrates during DNA replication is prevented, thus leading to progressive loss of 5mC in concert with cellular proliferation, and (B) active processes that remove the modification or modified bases from DNA without necessarily requiring DNA replication.^{97–102}

The discovery of abundant amounts of 5hmC in mammalian DNA together with the methylcytosine oxygenase enzymes responsible for its generation had a dramatic impact on the DNA methylation field—it required a revision of many cherished hypotheses and provided a multitude of new possibilities for the impact of DNA modifications on genome regulation, organization and integrity.^{56, 57, 97, 103}

By using thin layer chromatography (TLC), high pressure liquid chromatography (HPLC) and mass spectrometry (MS), Kriaucionis and Heintz detected the presence of 5hmC in Purkinje cells and granule cells comprising approximately 0.6% and 0.2% of total nucleotides, respectively.⁵⁶

Coinciding with this observation, Rao and coworkers discovered a key player responsible for active DNA demethylation, the Ten-Eleven-Translocation (TET) gene, Tet1. They found that TET1 can catalyze the conversion of 5mC to 5hmC in a 2-oxoglutarate (2OG) and Fe(II)-dependent manner.⁵⁷

Soon after, two other TET family members, TET2 and TET3, were also identified as being able to catalyze a similar reaction (Fig. 2.1B).¹⁰⁴ 5hmC can be generated from 5mC and subsequently further oxidized to 5-formylcytosine (fC) and 5-carboxylcytosine (caC) through the action of the TET enzymes (Fig. 2.1C).^{58, 104}

This pathway is implicated in enzymatic DNA demethylation processes during development and in disease states including response to environmental exposure.^{58, 105–108}

TET family proteins (TET1, TET2 and TET3) share a conserved C-terminal catalytic domain of Cys-rich and DSBH regions with homology to the dioxygenase superfamily.⁵⁸

Their potential redundancy adds an additional degree of complexity to unravelling the role of TET proteins and 5hmC in epigenetic regulation during development. Several possible DNA replication-independent active DNA demethylation pathways were inferred from in vitro studies; TET-catalysed 5hmC conversion into 5fC and 5caC can be efficiently excised from DNA by the DNA repair enzyme thymine-DNA glycosylase (TDG).¹⁰⁹

Subsequent repair of the resulting abasic site via base excision repair (BER) can generate an unmodified cytosine residue, thus completing the process of DNA demethylation.^{109, 110}

TDG possesses robust excision activity towards 5fC- and 5caC-containing DNA, but not 5hmC- and 5mC-containing DNA.^{109, 111}

Another possibility is that 5fC and 5caC can be directly deformylated or decarboxylated by a putative DNA deformylase or DNA decarboxylase enzyme, which eventually leads to the restoration of unmodified cytosine.¹¹²

Intriguingly, 5caC decarboxylase activity has been detected in mouse embryonic stem cell (mESC) lysates, but no putative decarboxylase has yet been identified.¹¹³

The first in vitro evidence for direct enzymatic decarboxylation of 5caC to unmodified cytosine demonstrated that IDCases (isocitrate decarboxylases) possess decarboxylase function albeit with weak activity.¹¹⁴

Another study found that C5-MTases (cytosine-5-S-adenosylmethionine-dependent DNA

methyltransferases) can convert 5caC directly to unmodified cytosine in vitro but are lacking this ability toward 5fC.¹¹⁵ The existence of 5fC DNA deformylase and 5caC DNA decarboxylase is uncertain. Alternatively, 5mC or 5hmC can be deaminated by cytidine deaminases, leading to replacement of the residue with an unmodified cytosine residue and resulting in active DNA demethylation. The activation-induced cytidine deaminase(AID)/apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) family of cytidine deaminases are well characterized for their ability to deaminate 5mC or 5hmC to generate thymidine (T) or 5-hydroxymethyluracil(5hmU), respectively. As these are present in mismatched T:G and 5hmU:G base pairs, they have been suggested to be excised by TDG or single-strand-selective monofunctional uracil DNAglycosylase1(SMUG1), another DNA glycosylase, leaving intact abasic sites in DNA. The resulting abasic sites are repaired by BER to restore unmodified cytosines.¹¹⁶

On the other hand, a recent study presented in vitro evidence that the mammalian de novo DNA methyltransferases DNMT3A and -3B, in addition to their well-known methyltransferase activity, could also function as reduction-oxidation (redox) state-dependent DNA dehydroxymethylases that can convert 5hmC directly to unmodified cytosine, although the exact pathways are yet to be better defined and the physiological relevance has yet to be demonstrated.^{117, 118}

Mapping of the relatively stable 5hmC mark indicated it could have additional functions in active transcription and genome stability.^{119–122}

Delineating the precise roles of TET proteins in development is still ongoing, for example *Drosophila melanogaster* has a reduced repertoire of DNA methyltransferases (only DNMT2)and barely detectable amounts of 5mC present in its genome, yet it contains a highly conserved TET homologue (dTet) that regulates 5hmC levels in mRNA.¹²³ The dTet homologue is essential for fly development, perhaps by mediating mRNA translation efficiency of selected transcripts in different tissue contexts.¹²⁴

In an ES cell model, it has been recently shown that TET2 catalyses the 5hmC modification of MERV1 retrotransposon-derived RNAs resulting in their destabilization; suggesting that potentially transcriptionally active endogenous retrovirus RNAs can be modulated by resident TET enzymes.¹²⁵

2.4 DNA METHYLATION IN EARLY DEVELOPMENT

In early embryo development and during primordial germ cell (PGC) establishment, DNA methylation undergoes extensive reprogramming. Blastocyst epigenetic programming is under the control of signal induction pathways; this can be mimicked to a certain extent in mouse ES cell models grown in precise media conditions (Fig. 2.3)^{47,49, 50,93, 126, 127}.

DNA methylation reprogramming also occurs in somatic cell contexts, which are linked to cellular differentiation and cellular transformation processes.^{106,120,128} Developmental DNA reprogramming depends on a combination of DNA demethylation and remethylation pathways.^{47, 100, 126, 129, 130} TET enzyme conversion of 5-methyl cytosine may be one pathway of demethylation and its iron and α -ketoglutarate (α KG) dependent mechanism can be enhanced by the

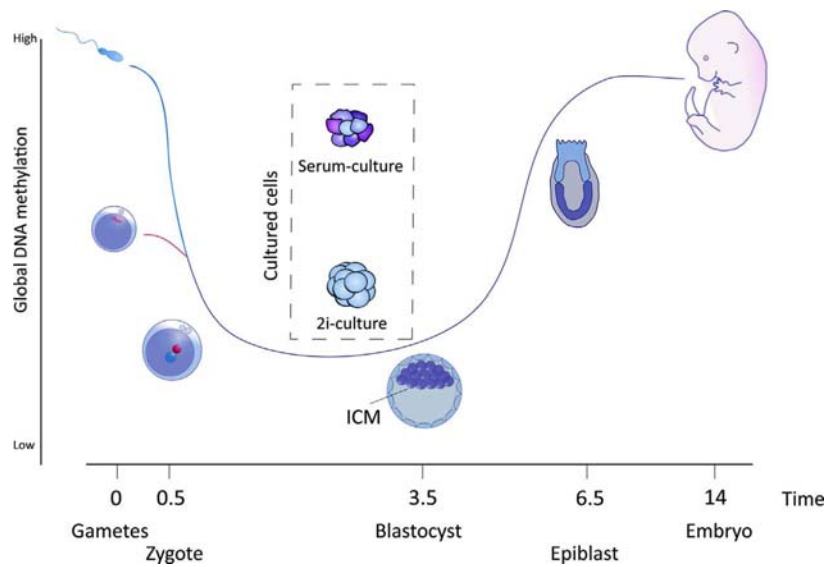


FIG. 2.3

Depiction of dynamic DNA methylation reprogramming during early mouse development, and ES cell culture models for signal-induced DNA methylation changes between metastable primed ES cells grown in serum/LIF and ground state ES cells grown in “2i” conditions, which are relatively hypomethylated.

addition of vitamin C.^{57,58,92,131} TET-mediated oxidation of 5mC can instigate both active and passive demethylation pathways. As oxidative derivatives are not well recognized by the methylation maintenance machinery, this may cause passive demethylation through DNMT1 enzyme inhibition after DNA replication.¹³² The presence of 5caC, or 5fC can also potentially trigger active erasure by the action of DNA glycosylases such as TDG, followed by BER.^{29, 58,}

^{109, 133} However, the contribution of TET-mediated demethylation to DNA methylation reprogramming pathways is context dependent. In mammals, active demethylation of sperm pronuclear DNA shortly after fertilization is thought to be important for subsequent zygotic gene activation and embryonic development.^{40, 134} The detection of 5hmC and its derivatives in zygotes suggested they may contribute to this process and TET3 was originally thought to be a possible mediator of zygotic demethylation.^{46, 99, 135–137} However, subsequent investigation demonstrated that the disappearance of 5mC and the appearance of 5hmC in the paternal genome may not be linked.¹⁰⁰

It is believed that this epigenetic reprogramming is an essential process that helps restore the totipotency in both zygotes and PGCs cells, thus permitting the cells to preserve their developmental potency and allowing the subsequent capability to differentiate into different cell lineages.¹³⁸ The first epigenetic reprogramming occurs in the early zygote stage, since both oocyte and spermatozoa are differentiated cells which need to be reprogrammed to restore the totipotency.¹³⁹ In mice, the DNA methylation levels in the male pronuclei rapidly diminish within 4 hours after fertilization, before the first cell division. The female pronuclei undergo a passive demethylation process due to the lack of a DNMT1 enzyme in the zygote, resulting in the failure of DNA methylation maintenance from parental strands to daughter strands during DNA replication.^{14, 15} In addition, despite the genome-wide erasure of DNA methylation, some regions of the genome are still methylated, including paternal imprinted genes, IAP

retrotransposons and some parts of heterochromatin that are situated around the centromere of the chromosome. These conserved methylated sequences are thought to help with the parental imprinted gene maintenance, IAP transposition repression, and maintaining chromosomal stability.¹⁴⁰

The discovery of the TET enzyme mechanism of oxidation of 5mC to 5hmC suggested a strong candidate for the active demethylation process in zygotes, as both TETs and 5hmC are found enriched especially in male pronuclei when compared with female pronuclei. In addition, the TET3 enzyme — not TET1, 2 — is highly expressed in oocytes and zygotes with the level later sharply reduced in the 2cell stage and gradually decreased during cleavage stages.¹⁴¹ 5mC in male pronuclei could therefore be actively demethylated by the TET3 enzyme and converted into 5hmC. An experiment with TET3 deficient zygotes derived from conditional knockout mice indicated that paternal-genome conversion of 5mC into 5hmC fails to occur and the level of 5mC remains persistent.¹⁴² While active demethylation by the TET3 enzyme occurs in mouse male pronuclei, DNA demethylation in female pronuclei remains constant and seems to escape this active demethylation. One potential mechanism for this protection from DNA methylation is through the proposed inhibition of the TET3 enzyme by PGC7 (or Dppa3 or Stella) protein.¹⁴³ Stella, which is found in female pronuclei through its association with H3K9me2 reduces the affinity of TET3 to these chromosomes resulting in low enzyme activity. Moreover, it is also believed that the little expression of Stella that is found in the male pronuclei is associated with the protection of the imprinted gene and chromosomal stability.¹⁴⁴ However, the hypothesis of 5mC conversion to 5hmC is not the only model for the DNA demethylation pathway in zygotes. Other reports propose that 5hmC is not merely the intermediate of the demethylation process, but instead has its own independent epigenetic role in development. Petra Hajkova's group revealed that in zygotes, the overall 5hmC expression is delayed and not synchronized with genome-wide 5mC demethylation.¹⁰⁰ The increase in 5hmC levels was only detected in PN4 stage zygotes after the major drop of 5mC level in the early PN3 stage, leaving a gap between PN2 to early PN3 stage where no 5mC nor 5hmC level was detected in male pronuclei. In addition, the group proposed that the expression of 5hmC is derived from de novo methylation by DNMT3 enzymes. The hypothesis was investigated by incubating a methyltransferase inhibitor with IVF zygotes, which resulted in a significant decline of 5hmC levels in the male pronuclei, whereas there was no significant difference in 5mC level in control and treated groups. This suggested that 5hmC deposition was not dependent on genome-wide 5mC demethylation.¹⁰⁰ Moreover, Iqbal et al., 2011 proposed that the demethylation of 5mC to 5hmC is not followed by the base excision oxidation pathway, since the 5hmC remains present after mitotic activity and through all cleavage stage embryos.^{141, 145} Collectively, these findings suggest that 5hmC is a stable marker and may possibly have its own role during early development.

The methylation level of the cleavage stages embryos is progressively reduced over the course of development until the blastocyst stage. The demethylation process during this stage occurs by passive demethylation. The DNMT1 oocyte variant (DNMT1o), which is inherited from the female and found in oocytes, disappears during cell division in zygotes.^{146, 147} This lack of DNMT1 is thought to account for the demethylation during cleavage stages.¹⁴⁸ In contrast, the DNA demethylation in PGCs shows a highly dynamic process. After their migration to the genital ridges, DNA methylation is rapidly erased at E13.5 despite the DNMT1 enzyme being available in the cells. The short time scale of the process in the presence of DNMT1 enzyme was suggested to be due to active demethylation.⁴¹ The demethylation

process in PGCs is thought to be responsible for erasing the parentally inherited imprinted genes, which were protected during the genome-wide demethylation in early development.^{149–151} It is also noteworthy that inactivation of TDG preferentially affects late stage embryonic development, which implies that it has no role in paternal demethylation.¹⁵² In early development, the bulk of 5mC, 5hmC, 5fC and 5caC appear to be lost by replication-dependent dilution. However, it is clear in many other developmental and disease contexts that inhibition of TET activities can be associated with 5mC accumulation by loss of either passive or active demethylation pathways, which may or may not have functional consequences^{153–157}. It should also be borne in mind that additional functions for TET enzymes have been identified that have yet to be fully explored.^{123, 125, 156, 158–161} Combinatorial analysis has shown that the patterns of DNA modifications vary greatly between tissue and cell types — to the extent that 5hmC profiling itself can be used as an exquisite identifier of cell state or tissue type as it is preferentially present at the gene bodies of expressed genes.^{20, 39, 162–164} A consensus view is that 5hmC-modified DNA modification patterns can thus act as identifiers of cell state. Proximal enrichment of 5hmC at enhancers upstream of annotated transcriptional start sites (TSS) suggests a role for these regions in the regulation of gene expression in development.^{119, 120} Not surprisingly, the enhancer related marks, H3K4me1/H3K27ac, are associated with 5hmC at upstream regions flanking transcription start sites (TSS).^{120, 165}

2.5 HOW NECESSARY IS DNA METHYLATION REPROGRAMMING FOR EARLY DEVELOPMENT?

Early studies demonstrating that the maintenance (DNMT1) and de novo methyltransferases (DNMT3A and DNMT3B) are required for mouse development to differing extents strongly implied that DNA methylation is essential to complete this process; the inactivation of DNMTs could be associated with some misexpression, especially of endogenous retrotransposons in post-blastocyst E9.5 embryos (Table 2.1)^{60, 61, 176}.

In this capacity it is required primarily for subsequent heritable transcriptional repression of retrotransposons, maintenance of monoallelic expression of imprinted genes and for the maintenance of X chromosome inactivation in female cells.²⁵ However, charting the dynamics of DNA methylation suggests that mouse blastocysts are significantly hypo-methylated, implying that DNA methylation is also required for postblastocyst embryos as differentiation occurs, perhaps to ensure stable memory of differentiated expression states (Fig. 2.4).^{15, 47, 80, 177} Pluripotent embryonic stem (ES) cells that are derived from blastocysts adopt an alternative epigenetic constitution in culture, but these primed ES cells can be reprogrammed to a more naïve epigenetic state reminiscent of the hypomethylated inner cell mass (ICM) when cultured with inhibitors of two kinases (Mek and GSK3), known as “2i”.^{93, 178, 179} However, it should be noted that ES cells lacking maintenance and de novo methyltransferases (TKO cells) grown in standard medium can self-renew and are indistinguishable from their wild-type counterparts as primed stem cells; it is only upon differentiation that they lose out in competition with their wild-type counterparts.¹⁸⁰ Sakaue and colleagues subsequently showed by nuclear transfer experiments that hypomethylated nuclei from TKO ES cells could support mouse preimplantation development until the blastocyst stage in an equivalent fashion to WT ES nuclei.¹⁶⁸ Subsequently few TKO cells were observed in the embryo proper (which is normally methylated), but they did contribute to normally hypomethylated extra-embryonic tissues. This was validated by in vitro studies in which TKO ES underwent apoptosis during their differentiation into

Table 2.1 Phenotypes of Mutated Epigenetic Modifiers in ES Cells and Mice			
Dnmts	KO ES Cells	Homozygous KO Embryo	Ref.
Dnmt1	Self-renew	Growth retarded after E6.5, lethal before E12.5	60
Dnmt3a	Self-renew	Lethal postnatally	61
Dnmt3b	Self-renew	Lethal post E9.5	61
Dnmt3a/Dnmt3b DKO	Self-renew	Lethal before E9.5	61
Dnmt1/Dnmt3a/ Dnmt3b TKO	Self-renew	Nuclear transfer experiments show development supported up to blastocyst stage	166
Dnmt3L	Self-renew	Altered germ cell development	63
Dnmt3C	Not expressed	Impaired male germ cell development	65
Tets			
Tet1	Self- renew	Viable, fertile and grossly normal but some mutant mice are born with a slightly smaller body size	167
Tet2	Self- renew	Predisposition to myeloid leukemia	168
Tet3	Self- renew	Maternal KO causes neonatal sublethality	169
Tet1/Tet2 DKO	Self- renew	Some DKO embryos exhibit mid-gestation abnormalities with perinatal lethality. Viable and overtly normal DKO mice are also observed	154
Tet1/Tet2/Tet3 TKO	Self- renew	TKO embryos have gastrulation phenotypes, including primitive streak patterning defects; they mimic phenotypes in embryos with gain-of-function Nodal signaling	170
Other factors			
Tdg	Self-renew	Tdg-null embryos isolated up to embryonic day E10.5 appear alive and normal, but lethal before E12.5	171
Uhrf1 (NP95)	Self- renew	Homozygous mutants exhibit abnormal development	172, 173
Hells (Lsh)	Self- renew	Growth retardation and premature aging phenotypes in mice with Hells disruption	174, 175

epiblast lineages, but not extraembryonic lineages.¹⁶⁶ These observations suggest that extraembryonic lineage cells, like ES cells and embryos during preimplantation development, can survive and proliferate in the absence of DNA methyltransferases and that the impact of global hypomethylation is cell-type dependent. Mutation of other factors associated with the DNA methylation/demethylation pathways tends not to affect the capacity of ES cells to self-renew and results in later stage developmental defects in mice (Table 2.1). Remarkably, hypomethylated (TKO) trophoblast stem (TS) cells can misexpress genes normally repressed by DNA methylation, but this does not compromise their cellular identity.¹⁶⁶ The lack of dependence of ES cells and early embryos on DNA methylation and other repressive epigenetic mechanisms is an example of what has been described as an “epigenetic paradox”, in which early cell fate is determined primarily by pluripotency networks. Epigenetic mechanisms only come on line and become necessary

during differentiation, when specialized cellular functions are incompatible with pluripotent states (Figs.2.3 and 2.4)

¹⁸¹. Lsh/Hells is a putative helicase required to maintain methylation and silencing at interspersed endogenous retro viruses (ERVs).¹⁸² Lsh^{-/-} mutants die either at birth or later from a premature aging phenotype associated with cellular senescence; significantly, their genomes are globally DNA hypomethylated, indicating that significant global loss of DNA methylation is not a strict impediment to postblastocyst development even though many ERVs are misexpressed^{174, 183, 184}. Epigenetic regulation thus serves to reinforce cell fate decisions and maintain the genome integrity of committed cells over time; subsequent ongoing environmental exposure and age-associated changes can lead to cellular breakdown and malfunctioning homeostasis resulting in pathophysiologic states that are incompatible with life^{51, 163}.

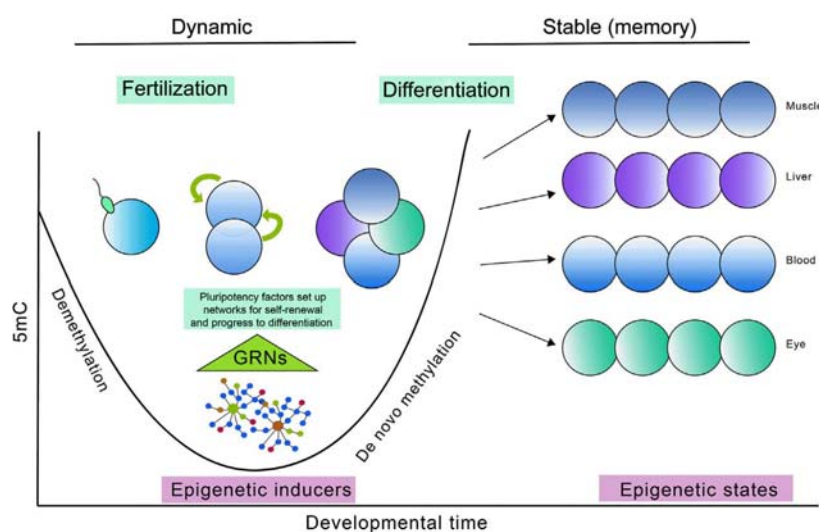


FIG. 2.4

General model illustrating that dynamic pluripotent states in low DNA methylation conditions in early development direct the formation of gene regulatory networks (GRN) that will specify and maintain stable differentiated states, which are reinforced by epigenetic pathways so that stable cell memory or identity is maintained during cell division.

2.6 DNA METHYLATION TRAJECTORIES DURING REPROGRAMMING OF SOMATIC CELLS

As differentiation proceeds, epigenetic barriers are set up to preserve tissue-specific demarcation states in somatic cells. Reversion to earlier pluripotency or trans-differentiation is incompatible with organ specific function but it is now recognized that tissue regeneration via stem cell activation can be forced under extreme physiological stress such as liver injury.¹⁸⁵ A combination of diet-induced liver injury along with inhibition of hepatocyte proliferation can result in significant regeneration of functional hepatocytes from biliary cells in a mouse model.¹⁸⁶ In addition, it is reported that laterally confined growth of fibroblasts on micropatterned substrates can induce nuclear reprogramming to stem-like cells with high efficiency in the absence of any exogenous reprogramming factors.¹⁸⁷ This process may replicate tissue niches that maintain stem cells for potential utilization in various organs. Expression analysis confirmed there

was a systematic progression from a mesenchymal to a stem cell-like transcriptome during this progression to a stem-like state over ten days.¹⁸⁷ In mammals, the first demonstration of reversion of a somatic cell to pluripotency was via somatic cell nuclear transfer experiments in sheep, which could give rise to cloned lambs derived from the nuclei of differentiated foetal and adult cells.^{18, 188} Subsequent immunostaining experiments suggested that sheep fibroblast somatic nuclei were partially demethylated upon transfer to recipient sheep oocytes and underwent a stepwise passive loss of DNA methylation during early development that is not as extreme as observed in early mouse embryogenesis.^{189, 190} Between the eight-cell and blastocyst stages, DNA methylation levels in sheep nuclear transfer embryos are comparable with those derived in vivo, but the distribution of methylated DNA is abnormal in a high proportion, probably as a consequence of failed nuclear reorganization of somatic heterochromatin.¹⁸⁹ Both remodeling of DNA and epigenetic reprogramming appear therefore critical for development of both fertilized and nuclear transfer embryos. Cloned mice could also be achieved by somatic cell nuclear transfer with success rates partially determined by the nuclear donor tissue type.^{191, 192} Successful reprogramming to generate live animals correlated with activation of pluripotency networks and normal DNA methylation dynamics.^{191, 193} The development of in vitro reprogramming methodologies to transform somatic cells to a state resembling pluripotency revolutionized the regenerative medicine field and prompted the development of direct somatic cell state conversion methodologies to other differentiated cell types.^{19, 194, 195}

Takahashi and Yamanaka first demonstrated that induced pluripotent stem cells (iPSCs) could be generated over time via the ectopic expression of only four transcription factors (TFs): OCT4, SOX2, KLF4 and c-MYC.^{19, 195} This so-called OSKM cocktail can convert embryonic and adult fibroblasts to iPSCs that can subsequently support mouse development. Many laboratories used the original protocol as the basis for generating iPSCs from diverse species and cell types and subsequently used these as models for early development and in vitro differentiation.^{194–196} Essentially these experiments illustrated that epigenetic barriers are removable, as reprogramming by TFs converts somatic cells with a distinct somatic epigenetic profile to one resembling a pluripotent state in iPSCs.^{20, 164, 197}

In standard OSKM conversion protocols, iPSC-like colonies can appear 7–10 days after OSKM activation and a number of studies have generated molecular profiles (expression and epigenetic states) of transition states from the originating somatic cell to the iPSC cells that can support mouse development.^{21, 197–199} These studies have identified unique trajectories that facilitate conversion of somatic cells to iPSCs.^{91, 198, 200–203} For example global levels of 5-methylcytosine fluctuate during conversion, which would be predicted to direct effects on chromatin organization and enhancer usage (Fig. 2.5).^{91, 203, 204} This may occur independently of transcriptional activation of pluripotency markers and specific changes at regulatory regions (Fig. 2.5).⁹¹ Expression analysis suggests there are surges in the upregulation of pluripotency genes, and transient bursts of upregulation of genes associated with alternative tissue types.¹⁹⁸ This implies that OSKM reprogramming may take advantage of several energetically or biologically favorable routes to pluripotency that do not necessarily represent a simple reversal of developmental processes, which by their nature are species specific. Not surprisingly, early transcriptional events orchestrated by the OSKM network correlate with a general relaxation of somatic chromatin states.²⁰⁴ This probably facilitates passage to a combined chromatin environment and gene

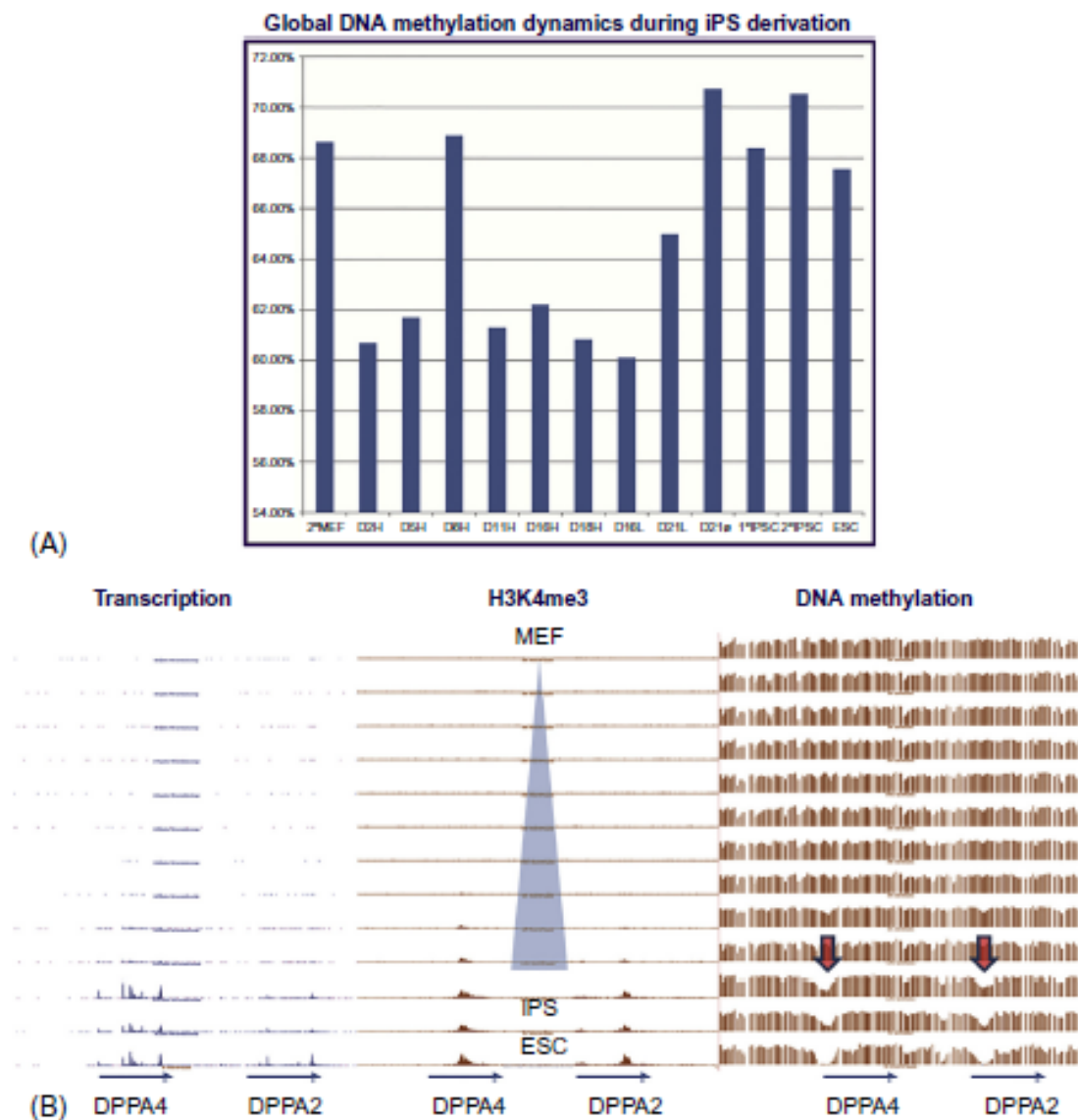


FIG. 2.5

(A) The global changes in 5mC levels are plotted during secondary reprogramming of mouse embryonic fibroblast to induced pluripotent stem cells (iPSCs); various transition states were captured in the study by Nagy and colleagues⁹¹. (B) Transcriptional activation of the developmental pluripotency associated factors DPPA4 and DPPA2 occurs late in OSKM reprogramming of fibroblasts and is coincident with the occurrence of the activating mark, H3K4me3, and hypomethylation at their promoters (red arrows). The order of the samples is the same as the 5mC analysis in (A). The genome browser data is derived from the Project Grandiose portal at <http://www.stemformatics.org>.

regulatory network that opens up a route to stem cell fates. It would thus be not surprising if epigenetic signatures could potentially impact on reprogramming. Loss of function studies suggested that conditional deletion of the de novo methyltransferase's, Dnmt3a and Dnmt3b, in fibroblasts did not impact on iPSC generation, however, in this study the effects on global methylation levels and chromatin organization were not examined.²⁰⁵ In contrast, ablation of the

methylcytosine dioxygenases Tet1-3, in embryonic fibroblasts inhibits their reprogramming to iPSCs because of a block in the mesenchymal to-epithelial transition (MET) step.²⁹ In embryonic fibroblasts (MEFs), TET-dependent reactivation of a set of miRNAs (miR-200 family) is essential for reprogramming; in contrast, neonatal keratinocytes can be reprogrammed efficiently upon conditional deletion of the only functional Tet3 allele, while neural progenitor cells (NPC) lacking TET enzymes can also be efficiently reprogrammed.²⁹ This suggests that tissue-specific epigenetic profiles influence reprogramming trajectories. In addition, Tet1 is highly expressed in ESCs and can replace multiple transcription factors during somatic cell reprogramming to generate high-quality mouse iPSCs in concert with the OCT4TF.^{206, 207} It will be interesting to know in future if hypomethylated embryonic fibroblasts have altered reprogramming trajectories to an iPSC state.

2.7 6-METHYLDEOXYADENOSINE IN MAMMALIAN DNA?

A potential new addition to the DNA modification profile of mammals is 6-methyldeoxyadenosine (6mA), which has been intensively studied in bacteria and was more recently characterized in invertebrates. In bacteria, 6mA was identified as a component of restriction-modification (R-M) systems that methylate target recognition sites to make these refractory to digestion by restriction enzymes.²³ This is part of a bacterial host-defense mechanism against infection/invasion by non-modified phages and plasmids. In 2015 6mA was identified at various levels of abundance in *Chlamydomonas*, *C. elegans*, and *Drosophila*, with the suggestion that it has a gene regulatory function.^{208–211} Despite ongoing research efforts to detect its potential presence and function in mammals and other vertebrates, the existence of 6mA in mammals remains controversial.^{212–214} A recent ultrasensitive LC-MS2-based approach, using an isotopically labelled m6dA standard, was unable to confirm the presence of m6dA in vertebrates.²¹⁵ This impacts on the potential for detecting 6mA by sequencing methodologies, including real-time sequencing where the less abundant a modification the greater the coverage is needed to detect its signal above background, especially when using target sequence enrichment as part of the analysis pipeline.²¹⁶ DNA immunoprecipitation with modification-specific antibodies followed by sequencing (DIP-seq) was recently shown to have an intrinsic background due to the affinity of IgG for short unmodified DNA repeats, which becomes even more problematic if the modification being assayed has very low abundance.²¹⁷ Genome-wide 6mA DIP-seq in mice can generate profiles nearly identical to those observed with random IgG antibodies on DNA samples lacking the target modifications.²¹⁷ Matched input and DIP-IgG sequencing controls can overcome this background effect and enable improved data interpretation, especially if complementary non-antibody-based techniques are also used to validate DIP-based findings. Further investigation is needed of the relevance of 6mA in mammalian DNA. In contrast, the contribution of 6-methyladenosine to RNA biology (stability, splicing and targeting) seems robust.²¹⁸

2.8 FUTURE PERSPECTIVES

Future studies should concentrate on dissecting the cause-consequence relationships of epigenetic systems in development, and the role of epigenetic plasticity in driving cellular transitions and in maintaining cell identity. Finally, the availability of new gene-editing tools and single-cell sequencing studies may enable exquisite investigation of the functional importance of developmental epigenetic profiles, especially when allied with novel in vitro proxies for in vivo development.^{219–223}

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